Airway Inflammation and Responsiveness in Prostaglandin H Synthase–Deficient Mice Exposed to Bacterial Lipopolysaccharide

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Bacterial lipopolysaccharide (LPS) is a risk factor for exacerbation of asthma and causes airway inflammation. The aim of this study was to examine the effects of disruption of prostaglandin (PG) H synthase (PGHS)-1 and PGHS-2 genes on pulmonary responses to inhaled LPS. PGHS-1$^{-/-}$, PGHS-2$^{-/-}$, and wild-type (WT) mice were exposed to 4 to 6 μg/m^3 LPS via aerosol. Enhanced pause (PenH), a measure of bronchoconstriction, was assessed using a whole-body plethysmograph before and immediately after a 4-h LPS exposure. Bronchoalveolar lavage (BAL) was performed after LPS exposure to assess inflammatory cells, cytokines/chemokines (tumor necrosis factor-α, interleukin-6, and macrophage inflammatory protein-2), and PGE_2. The degree of lung inflammation was scored on hematoxylin-and-eosin–stained sections. PGHS-1 and PGHS-2 protein levels were determined by immunoblotting. All mice exhibited increased PenH and methacholine responsiveness after LPS exposure; however, these changes were much more pronounced in PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice relative to WT mice ($P < 0.05$). There were no significant differences in inflammation as assessed by BAL fluid (BALF) cells or lung histology between the genotypes despite reduced BALF cytokines/chemokines and PGE_2 in PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice relative to WT mice ($P < 0.05$). PGHS-2 was upregulated more in PGHS-1$^{-/-}$ mice compared with WT mice after LPS exposure. We conclude that: (*1*) airway inflammation and hyperresponsiveness are dissociated in PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice exposed to LPS; (*2*) the balance of PGHS-1 and PGHS-2 is important in regulating the functional respiratory responses to inhaled LPS; and (*3*) neither PGHS-1 nor PGHS-2 is important in regulating basal lung function or the inflammatory responses of the lung to inhaled LPS.

Endotoxins are heat-stable lipopolysaccharide (LPS) protein complexes that are part of the outer membrane of gram-negative bacteria. They are potent proinflammatory substances that are present in a variety of domestic and occupational dusts (1). LPS is an important determinant of asthma severity, particularly in allergic patients exposed to high levels of allergen (2). It has also been suggested that chronic exposure to inhaled LPS is associated with the development and/or severity of occupational lung diseases, including byssinosis and airway disease induced by agricultural dusts (1). In rodents and humans, acute inhalation of LPS induces a severe lung inflammatory response characterized by activation of alveolar macrophages, recruitment of polymorphonuclear leukocytes (PMNs) into the airway, and release of proinflammatory cytokines (e.g., tumor necrosis factor [TNF]-α, interleukin [IL]-1, IL-6, and IL-8), chemokines (e.g., macrophage inflammatory protein [MIP]-2), eicosanoids (e.g., leukotriene [LT] B_4), reactive oxygen species, cytolytic proteases, and lysozomal enzymes (3–5). The inflammation is accompanied by increased vascular permeability, bronchoconstriction, and bronchial hyperresponsiveness (3, 4, 6).

Arachidonic acid (AA) is metabolized to bioactive eicosanoids that affect lung function. A wealth of data suggests that the LTs, products of the arachidonate 5-lipoxygenase (5-LO) pathway, are proinflammatory mediators that cause airway inflammation, bronchoconstriction, and increased lung vascular permeability (7). The role of prostaglandin (PG) H synthase (PGHS)–derived eicosanoids in the lung is less clear. Both PGD_2 and $PGF_{2\alpha}$ cause bronchoconstriction (8, 9). In contrast, PGE_2 has bronchodilatory effects, and blocks both the early and late asthmatic responses to allergen challenge (9). Prostanoids of the E-series and their analogs have also been shown to inhibit recruitment of inflammatory cells into the lung, decrease their survival, and inhibit their activation (10, 11). Two distinct PGHS enzymes have been described (12). PGHS-1 is believed to be a "housekeeping" enzyme that produces PGs which are required for maintenance of normal cell/organ function. In contrast, PGHS-2 is an inducible enzyme that is upregulated by cytokines and phorbol esters, highly expressed in inflamed tissues, and believed to produce PGs involved in inflammatory processes (12).

Recently, mice lacking either the *Pghs-1* or *Pghs-2* gene have been constructed using gene-targeting strategies (13, 14). PGHS-1$^{-/-}$ mice exhibit decreased AA-induced ear inflammation compared with wild-type (WT) mice, whereas PGHS-2$^{-/-}$ mice have normal ear inflammatory responses to phorbol ester and AA (13, 14). We have demonstrated that, at baseline, there are no significant differences in lung function or lung histopathology between PGHS-1$^{-/-}$, PGHS-2$^{-/-}$, and WT mice (15). In contrast, allergic lung inflammatory responses are markedly increased in PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice relative to WT mice (15). Interestingly, whereas both allergic PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice exhibit decreased total respiratory system compliance, only allergic PGHS-1$^{-/-}$ mice show increased total respiratory system resistance and responsiveness to methacholine (MCh)

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Abbreviations: arachidonic acid, AA; airway hyperresponsiveness, AHR; bronchoalveolar lavage, BAL; BAL fluid, BALF; interleukin, IL; 5-lipoxygenase, 5-LO; lipopolysaccharide, LPS; leukotriene, LT; methacholine, MCh; macrophage migration inhibitory factor, MIF; macrophage inflammatory protein, MIP; messenger RNA, mRNA; enhanced pause, PenH; prostaglandin, PG; PG H synthase, PGHS; polymorphonuclear leukocyte, PMN; radioimmunoassay, RIA; ribonuclease protection assay, RPA; tumor necrosis factor, TNF; wild-type, WT.

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(15). Hence, allergic airway inflammation can be dissociated from the development of airway hyperresponsiveness (AHR) in the PGHS-$2^{-/-}$ mice.

PGHS inhibitors (such as aspirin and indomethacin) enhance lung neutrophil recruitment and cytokine production after inhalation of LPS in mice and hamsters (16). Moreover, pretreatment with PGE_2 blocks LPS-induced lung inflammatory responses, suggesting that PGHS-derived eicosanoids limit lung inflammation after LPS exposure (16). To better define the relative roles of the two PGHS enzymes and their bioactive eicosanoid products in the pathogenesis of lung inflammation and AHR, we used PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice in an established model of airway disease in which animals are exposed to inhaled LPS. Hence, the objectives of this study were to examine whether PGHS-1 and/or PGHS-2 isoforms contribute to the airway inflammatory and functional responses to inhaled LPS.

Materials and Methods

Experimental Animals

Male and female, pathogen-free, 5 to 7-mo-old PGHS-$1^{-/-}$, PGHS-$2^{-/-}$, and littermate WT control mice were of a hybrid C57BL/6J × 129/Ola genetic background intercrossed for 15 to 20 generations and bred at NIH/NIEHS. Mice were genotyped using a combination of polymerase chain reaction and Southern blotting of tail DNAs using previously published methods (13–15). All animal care and housing requirements set forth by the NIH Committee on Care and Use of Laboratory Animal Resources were followed. Animal protocols were approved by the Institutional Animal Care and Use Committee. Food and water were supplied *ad libitum* throughout the experiment.

Experimental Protocol

Mice were exposed to *Escherichia coli* 0111:B4 LPS (Sigma, St. Louis, MO) by aerosol (4 to 6 $\mu g/m^3$) for 4 h in a glass 75-liter exposure chamber using a Collision nebulizer (BGI, Waltham, MA) at a flow rate of 10 to 17 ml/min as described (4). The chamber atmosphere was exchanged at a rate of 1 exchange/min. In some animals, lung function measurements were made before and immediately after LPS exposure. Other mice were killed immediately after LPS exposure or 24 h later. These time points were chosen because previous studies in our laboratories and others indicate that lung inflammation and bronchial hyperresponsiveness are maximal approximately 4 h after LPS exposure and decline 24 h later (4, 16). The chest was opened and both lungs were lavaged *in situ* by instillation and withdrawal of 6 × 1 ml sterile pyrogen-free saline. After bronchoalveolar lavage (BAL), the left lung was perfusion-fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. The right lung was snap-frozen in liquid nitrogen and stored at − 80°C.

Lung Function Measurements

The enhanced pause (PenH), a measure of bronchoconstriction, was assessed at baseline and after increasing doses of aerosolized MCh (12.5 to 50 μg/kg) using a Biosystem XA whole-body plethysmograph (BUXCO, Sharon, CT) as described (17, 18). Briefly, animals were placed in an 80-ml plethysmograph ventilated by bias airflow at 0.2 liter/min. The breathing patterns and pulmonary function of each mouse were monitored over time with direct measurements of respiratory rate, box pressure, and box flow. Airway resistance was expressed as PenH = (expiratory time/40% of relaxation time − 1) × peak expiratory flow/peak inspiratory flow × 0.67. The use of PenH as a measure of bronchoconstriction has been previously validated (17–19).

Analysis of BAL Fluid

BAL fluid (BALF) was placed on ice and centrifuged at 200 × *g* for 5 min at 4°C. Supernatants were decanted and frozen at − 80°C for subsequent use. The cell pellet was resuspended, washed twice in Hanks' balanced salt solution, and counted using a hemocytometer. Slides of BALF cells were prepared using a Cytospin-2 cytocentrifuge, stained with Diff Quick and differentiated using conventional morphologic criteria. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used for measurement of murine TNF-α, IL-6, and MIP-2 (R&D Systems, Minneapolis, MN) in cell-free BALF according to the manufacturer's instructions. Levels of PGE_2 and LTB_4 were determined by radioimmunoassay (RIA) using kits supplied by Amersham Life Sciences (Arlington Heights, IL) as described (15). Total protein levels were measured using the method of Branford (20) with reagents purchased from Bio-Rad (Richmond, CA).

Lung Histopathology

Serial sections (5 to 6 μm) were stained with hematoxylin and eosin (H&E) and Alcian blue/periodic acid-Schiff. A semiquantitative histopathologic scoring system was developed on the basis of the presence and abundance of the following: (*1*) perivascular edema (0, absent; 1, mild to moderate, involving fewer than 25% of the perivascular spaces; 2, moderate to severe, involving more than 25% but less than 75% of perivascular spaces; or 3, severe, involving more than 75% of perivascular spaces); (*2*) perivascular/peribronchial acute inflammation (0, absent; 1, mild acute inflammation in the perivascular edematous space, with fewer than 5 neutrophils per high-power field [hpf]; 2, moderate acute inflammation in the perivascular spaces, extending to involve the peribronchial spaces, with more than 5 neutrophils per hpf in these regions; or 3, severe, acute inflammation in the perivascular and peribronchial spaces with numerous neutrophils encircling most [> 50%] of bronchioles); (*3*) goblet-cell metaplasia of bronchioles (0, absent; 1, few goblet cells present in one or two bronchiolar profiles; or 2, large numbers of goblet cells present); and (*4*) eosinophilic macrophages in alveolar spaces (0, absent; 1, present in fewer than 25% of alveolar spaces; or 2, present in > 25% of alveolar spaces). A total inflammatory score (range 0 to 10), taken as the sum of the individual scores, was determined by a pulmonary pathologist who was blinded to genotype and treatment-group assignment.

Western Blotting

Whole-lung lysates were prepared from frozen lung tissues by homogenization in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM ethyleneglycol-*bis*-(β-aminoethyl ether)-*N,N′*-tetraacetic acid, 0.25% sodium deoxycholate, 1 mM NaF, 0.25 M phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml pepstatin, and 100 mM Na_3VO_4. Goat antimouse PGHS-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit antimouse PGHS-2 (Cayman Chemical Co., Ann Arbor, MI) were specific for their respective PGHS isoforms and used according to the manufacturers' instructions. Recombinant PGHS-1 and PGHS-2 protein standards were prepared as described (15). For immunoblotting, proteins were resolved by electrophoresis in 10% sodium dodecyl sulfate (SDS) polyacrylamide gels (80 × 80 × 1 mm) (Novex, San Diego, CA) and transferred electrophoretically to nitrocellulose membranes. Membranes were immunoblotted with the primary antibodies, goat antirabbit or rabbit antigoat immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad Laboratories) and visualized with the ECL Western Blotting Detection System (Amersham International, Buckinghamshire, UK).

Preparation of RNA and Multiprobe Ribonuclease Protection Assay

Total RNA was prepared from frozen lung tissue using RNA STAT-60 (Tel-Test, Friendswood, TX) as described (4, 18). Gene transcripts for TNF-α, IL-1β, IL-6, MIP-2, and macrophage migration inhibitory factor (MIF) were detected by ribonuclease protection assay (RPA) using probes purchased from Pharmingen (San Diego, CA) as described (4, 18). Protected products were separated on a 5% acrylamide–8 M urea gel and quantified by autoradiography. Equivalent amounts of RNA were examined as judged by the amount of L32, which encodes a ubiquitously expressed ribosomal subunit housekeeping protein.

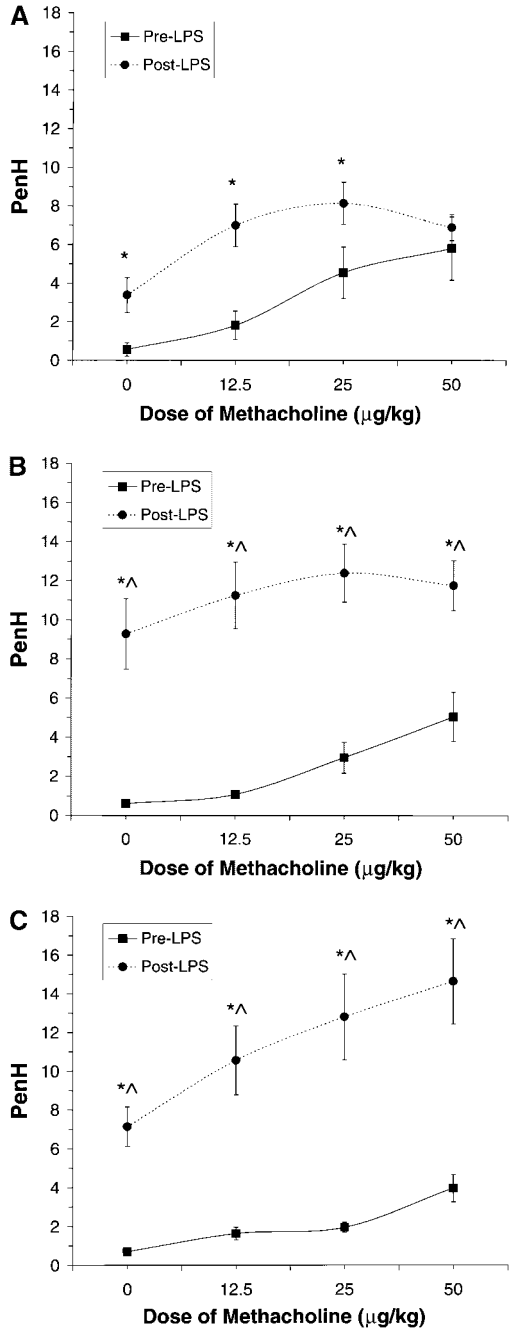

Figure 1. Baseline lung function and airway responsiveness to MCh in WT, PGHS-$1^{-/-}$, and PGHS-$2^{-/-}$ mice exposed to LPS. Mice (n = 8–9 animals per group) were exposed to 4 to 6 μg/m^3 LPS via aerosol for 4 h. Before and immediately after LPS exposure, PenH was measured at baseline and after increasing doses (12.5 to 50 μg/kg) of inhaled MCh. *$P < 0.05$ versus corresponding value before LPS exposure. ^$P < 0.05$ versus corresponding post-LPS exposure value in WT mice. (*A*) WT mice; (*B*) PGHS-$1^{-/-}$ mice; (*C*) PGHS-$2^{-/-}$ mice.

Statistical Analysis

All values are expressed as means ± standard error. Data were analyzed by analysis of variance using SYSTAT software. When P values indicated that a significant difference was present, Fisher's LSD test for multiple comparisons was used. Values were considered significantly different if $P < 0.05$.

Results

PGHS-Deficient Mice Have Increased Bronchoconstriction Immediately after LPS Exposure

Before LPS exposure, there were no significant differences between WT, PGHS-$1^{-/-}$, and PGHS-$2^{-/-}$ mice in baseline PenH or in responsiveness to inhaled MCh (Figure 1). Immediately after the 4-h LPS exposure, WT mice exhibited a significant 603% increase in baseline PenH compared with pre-exposure values (Figure 1A). Interestingly, these changes were much more pronounced in PGHS-deficient mice. Thus, immediately after LPS exposure, PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice exhibited 1,521% and 1,035% increases in baseline PenH, respectively (Figures 1B and 1C) ($P = 0.006$ for PGHS-$1^{-/-}$ and $P = 0.009$ for PGHS-$2^{-/-}$ versus WT). The LPS-induced bronchoconstriction was not significantly different between PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice. LPS exposure caused AHR to inhaled MCh in all groups, and these changes were also more pronounced in the PGHS-deficient mice. For example, at the 12.5-μg/kg dose of MCh, WT, PGHS-$1^{-/-}$, and PGHS-$2^{-/-}$ mice exhibited 386, 1,041, and 647% increases in PenH, respectively, compared with corresponding pre-exposure values (Figure 1) ($P = 0.02$ for PGHS-$1^{-/-}$ and $P = 0.05$ for PGHS-$2^{-/-}$ versus WT). The differences in lung function between the genotypes were no longer evident 24 h after LPS exposure, a time when postexposure PenH values were not significantly different from pre-exposure values (data not shown).

Airway Inflammatory Cell Influx Is Not Different in PGHS-Deficient versus WT Mice after LPS Exposure

We have previously shown that under basal conditions, there were no significant differences in the number or type of BALF cells in PGHS-deficient mice compared with WT mice (15). The large majority of cells (> 95%) were alveolar macrophages and < 1% were PMNs (15). Immediately after the 4-h LPS exposure, BALF from WT mice had 0.45 ± 0.07 million cells/ml, the large majority of which (> 94%) were PMNs (Figures 2A and 2B). Interestingly, both PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice had similar numbers of total BALF cells (0.38 ± 0.06 and 0.34 ± 0.04 million cells/ml, respectively; P = not significant [NS] versus WT) and BALF PMNs (85 and 88%, respectively; P = NS versus WT) immediately after LPS exposure (Figures 2A and 2B). Likewise, there were no significant differences be-

tween the genotypes in the number or type of BALF cells 24 h after LPS exposure (data not shown). Thus, although PGHS-deficient mice exhibited increased bronchoconstriction and MCh hyperresponsiveness compared with WT mice after LPS inhalation, the degree of LPS-induced airway inflammatory cell influx was similar between the genotypes.

Lung Histopathology Is Not Different in PGHS-Deficient versus WT Mice after LPS Exposure

After LPS exposure, WT mice exhibited marked lung histopathologic abnormalities, characterized by perivascular edema and perivascular/peribronchial acute inflammation (Figure 3A). The inflammatory infiltrate consisted primarily of neutrophils with a few mononuclear cells within the edematous expansion of the perivascular space or around the periphery of bronchioles. Both LPS-exposed PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice had qualitatively similar degrees of lung abnormalities (Figure 3A). A semiquantitative, histopathologic scoring system was developed to objectively evaluate the degree of lung inflammation in the different groups. The total score, taken as the sum of scores on four individual criteria, was determined by a pulmonary pathologist who was blinded to genotype and treatment-group assignment. There were no significant differences in lung histopathology score between WT, PGHS-$1^{-/-}$, and PGHS-$2^{-/-}$ mice immediately after the 4-h LPS exposure (Figure 3B). Similarly, no histopathologic differences were detected between the genotypes 24 h after LPS exposure (data not shown).

BALF Total Protein and Cytokine/Chemokine Levels after Inhalation of LPS

It has previously been demonstrated that inhalation of LPS alters pulmonary capillary permeability in laboratory animals (21). To determine whether there were differences in alveolar epithelial permeability between WT, PGHS-$1^{-/-}$, and PGHS-$2^{-/-}$ mice, we measured BALF total protein immediately after LPS exposure. BALF obtained from WT mice had 0.12 ± 0.01 mg/ml total protein. Both PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice had similar levels

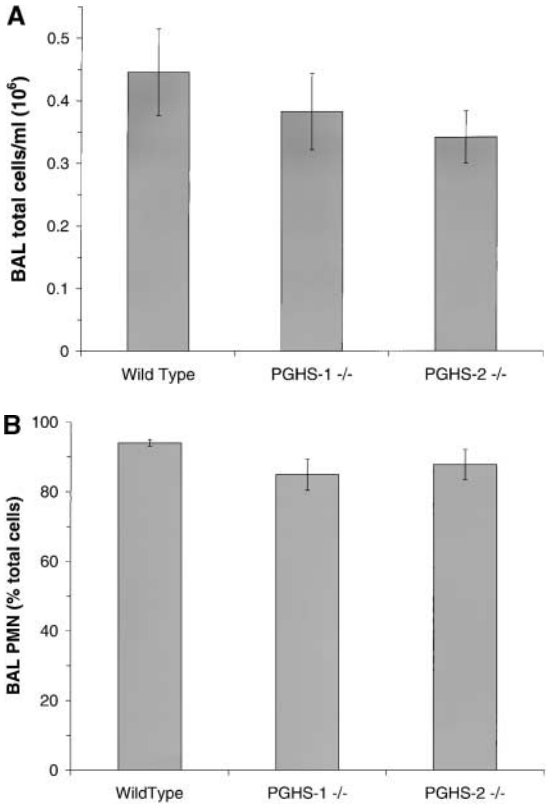

Figure 2. BALF cells in PGHS-$1^{-/-}$, PGHS-$2^{-/-}$, and WT mice exposed to LPS. Mice (n = 18–22 animals per group) were exposed to 4 to 6 $\mu g/m^3$ LPS via aerosol for 4 h. Immediately after LPS exposure, BAL was performed; cells were then counted using a hemocytometer and differentiated using conventional morphologic criteria. (*A*) Total BALF cells/ml; (*B*) percent of BALF cells that are PMNs.

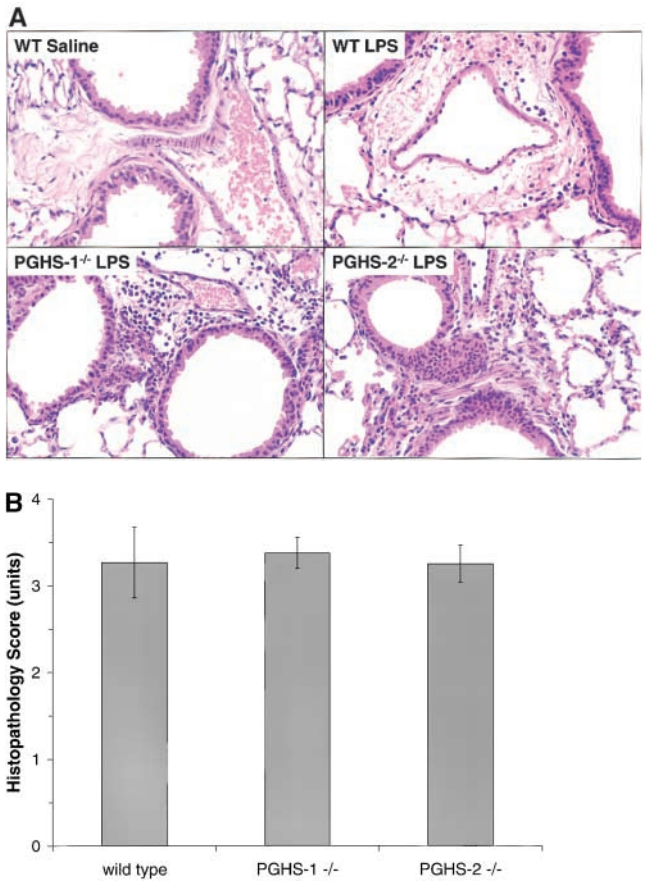

Figure 3. (*A*) Lung histopathology showing perivascular edema and perivascular/peribronchial acute inflammation in LPS-exposed WT, PGHS-$1^{-/-}$, and PGHS-$2^{-/-}$ mice. Mice were exposed to either saline (control) or 4 to 6 $\mu g/m^3$ LPS via aerosol for 4 h. After exposure, the left lung was fixed, embedded in paraffin, sectioned, and stained with H&E. Original magnification ×40. (*B*) Total histopathology score in PGHS-$1^{-/-}$, PGHS-$2^{-/-}$, and WT mice exposed to LPS. Mice (n = 12–16 animals per group) were exposed to 4 to 6 $\mu g/m^3$ LPS via aerosol for 4 h. Sections were prepared as described earlier and evaluated using a semiquantitative histopathologic scoring system as described in MATERIALS AND METHODS.

of BALF total protein (0.11 ± 0.02 and 0.11 ± 0.02 mg/ml, respectively; P = NS).

It has previously been shown that LPS causes significant increases in the concentration of several proinflammatory cytokines and chemokines in lavage fluid of mice, and that maximal levels occur within 4 h of LPS exposure (4). To determine whether LPS-induced changes in lung function were associated with differences in cytokine/chemokine levels, we measured TNF-α, IL-6, and MIP-2 by ELISA in BALF obtained from WT, PGHS-1$^{-/-}$, and PGHS-2$^{-/-}$ mice. Immediately after the 4-h LPS exposure, BALF from WT mice contained 1,216 ± 129 ng/ml TNF-α, 874 ± 152 ng/ml MIP-2, and 175 ± 30 ng/ml IL-6 (Figure 4). PGHS-1$^{-/-}$ mice had significantly lower BALF TNF-α levels (P = 0.0003) and tended to have lower BALF MIP-2 and IL-6 levels, although these differences did not reach statistical significance (P = 0.15 and 0.09, respectively) (Figure 4). In contrast, PGHS-2$^{-/-}$ mice had significantly lower BALF MIP-2 and IL-6 levels (P = 0.01 for both) and tended to have lower BALF TNF-α (P = 0.09) (Figure 4). Cytokine/chemokine concentrations in BALF were low to undetectable and not significantly different between the three genotypes 24 h after LPS exposure (data not shown).

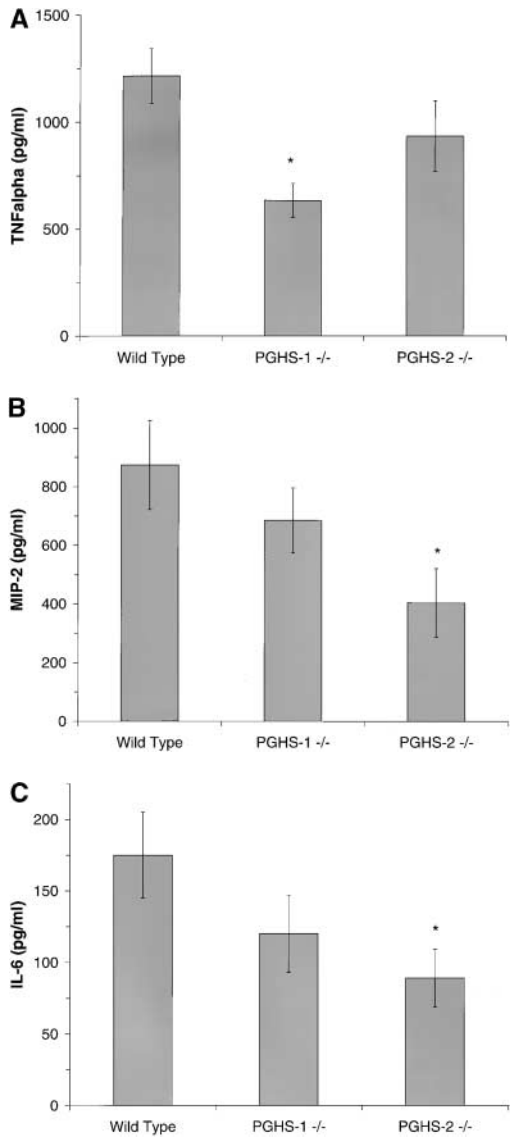

Figure 4. BALF cytokine levels in PGHS-1$^{-/-}$, PGHS-2$^{-/-}$, and WT mice exposed to LPS. Mice (n = 14–17 animals per group) were exposed to 4 to 6 μg/m^3 LPS via aerosol for 4 h. Immediately after LPS exposure, BAL was performed and the levels of TNF-α (*A*), MIP-2 (*B*), and IL-6 (*C*) were determined by ELISA as described in MATERIALS AND METHODS. *$P < 0.05$ versus corresponding value in WT mice.

To determine whether changes in cytokine/chemokine levels in BALF were accompanied by differences in total lung cytokine/chemokine messenger RNA (mRNA) expression, we performed RPAs using specific cellular RNA probes. In general, although the expression of mRNAs for TNF-α, IL-6, MIP-2, and other cytokines (e.g., IL-1β and MIF) was much greater immediately after the 4-h LPS exposure than 24 h later, there were no consistent or significant differences in cytokine/chemokine mRNA levels between the three genotypes (Figure 5).

Both PGHS-1 and PGHS-2 Contribute to Airway PGE_2 Biosynthesis after LPS Inhalation

We have previously shown that PGHS-1 is the major enzyme that biosynthesizes PGE_2 in mouse airways under basal conditions (15). To determine the relative contribution of the two PGHS isoforms to airway PG biosynthesis after LPS inhalation, we measured BALF PGE_2 levels by RIA in WT, PGHS-1$^{-/-}$, and PGHS-2$^{-/-}$ mice immediately after the 4-h LPS exposure. BALF from WT mice had 128 ± 10 pg/ml PGE_2. In contrast, both PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$

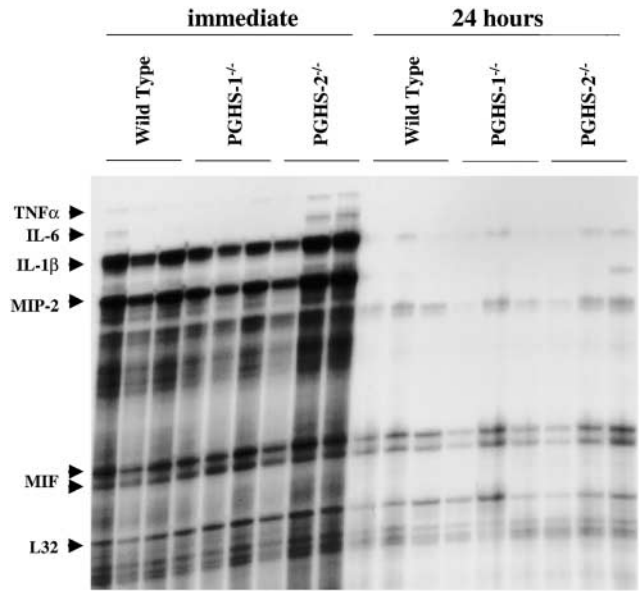

Figure 5. RPA of total RNA obtained from lungs of PGHS-1$^{-/-}$, PGHS-2$^{-/-}$, and WT mice exposed to LPS. Mice (n = 3 animals per group) were exposed to 4 to 6 μg/m^3 LPS via aerosol for 4 h. Immediately after LPS exposure or 24 h later, lungs were frozen and total RNA was assayed for expression of TNF-α, IL-6, IL-1β, MIP-2, and MIF mRNAs. Equivalent amounts of RNA were examined in each sample as evaluated by the amount of the housekeeping gene L32.

mice had significantly reduced BALF PGE_2 (43 ± 7 and 58 ± 8 pg/ml, respectively; $P = 0.0001$) (Figure 6A). This indicates that both PGHS isoforms contribute significantly to airway PGE_2 production after LPS exposure.

LT Biosynthesis Is Not Different in PGHS-Deficient Mice after LPS Inhalation

We have previously shown that PGHS-$1^{-/-}$ mice exhibit increased LT biosynthesis after allergen challenge, whereas PGHS-$2^{-/-}$ mice do not, suggesting that increased metabolism along the 5-LO pathway could contribute to allergen-induced inflammation and lung dysfunction in PGHS-$1^{-/-}$ mice (15). Given that LTs are potent bronchoconstrictors, and LTB_4 can increase adhesion of leukocytes to endothelial cells and is a potent chemotactic factor for neutrophils (5, 22, 23), we determined whether LT levels were altered in PGHS-deficient mice exposed to LPS. Interestingly, we found no significant differences in BALF LTB_4 concentrations between WT, PGHS-$1^{-/-}$, and PGHS-$2^{-/-}$ mice (Figure 6B). Hence, it is unlikely that increased 5-LO metabolism can account for the increased bronchoconstriction and hyperresponsiveness to MCh in LPS-exposed PGHS-deficient mice.

LPS Upregulates PGHS-2 Protein to a Greater Extent in PGHS-$1^{-/-}$ Mice

Protein immunoblotting with antibodies specific for PGHS-1 and PGHS-2 revealed that lungs from WT mice expressed both PGHS-1 and PGHS-2 proteins after LPS exposure, whereas PGHS-$1^{-/-}$ mice expressed only PGHS-2 protein and PGHS-$2^{-/-}$ mice expressed only PGHS-1 protein (Figure 7). Immediately after the 4-h LPS exposure, there was upregulation of lung PGHS-2 but not PGHS-1 protein. Interestingly, the degree of PGHS-2 upregulation was more pronounced in the PGHS-$1^{-/-}$ mice compared with WT mice (Figure 7). The magnitude of PGHS-2 upregulation was less pronounced 24 h after LPS exposure in both PGHS-$1^{-/-}$ and WT mice (Figure 7). Taken together, these data suggest that PGE_2 and/or other PGHS-1 products may limit LPS-induced upregulation of PGHS-2.

Discussion

The main purpose of this study was to define the relative functional roles of the two PGHS isoforms and their eicosanoid products in the pathogenesis of LPS-induced lung inflammation and airway dysfunction. Mice with disrupted *Pghs-1* or *Pghs-2* genes were used to circumvent potential problems associated with lack of isoform specificity of available PGHS inhibitors and the possible effects of these chemicals on unrelated metabolic pathways (12, 24). Indeed, published studies on the effects of PGHS inhibitors on LPS-induced airway inflammation and function have produced conflicting results. For example, Burrell and coworkers showed that indomethacin abolishes the increase in pulmonary capillary neutrophilia and blocks the decrease in lung volume that occurs in response to inhaled LPS (21). In contrast, de Moraes and colleagues found that both indomethacin and aspirin accentuate LPS-induced neutrophil recruitment and TNF-α production (16).

We found that all mice exhibited increased bronchoconstriction (PenH) immediately after LPS exposure; however, these changes were much more pronounced in PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice, relative to WT mice. Moreover, LPS-exposed PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice were more hyperresponsive to inhaled MCh than were LPS-exposed WT mice. We have previously shown that under basal conditions, there were no significant differences be-

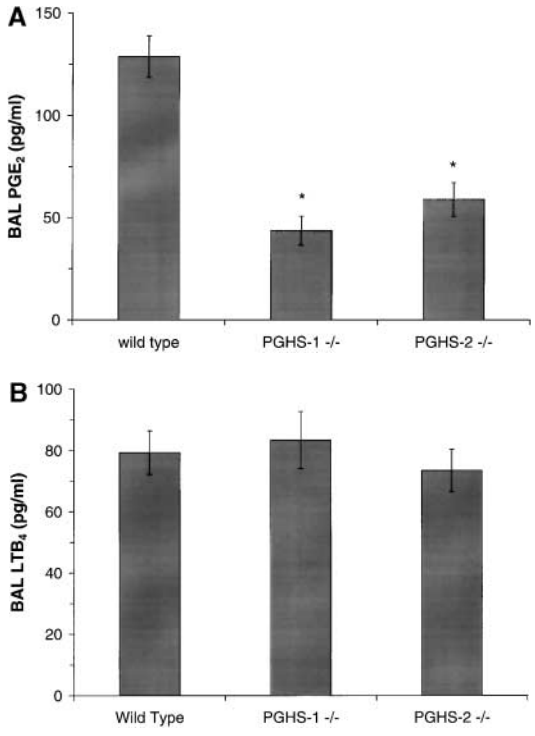

Figure 6. BALF eicosanoid levels in PGHS-$1^{-/-}$, PGHS-$2^{-/-}$, and WT mice. Mice (n = 12–16 animals per group for PGE_2; n = 7–8 animals per group for LTB_4) were exposed to 4 to 6 μg/m^3 LPS via aerosol for 4 h. Immediately after LPS exposure, BAL was performed and the levels of PGE_2 (*A*) and LTB_4 (*B*) were measured by RIA. *$P < 0.0001$ versus corresponding value in WT mice.

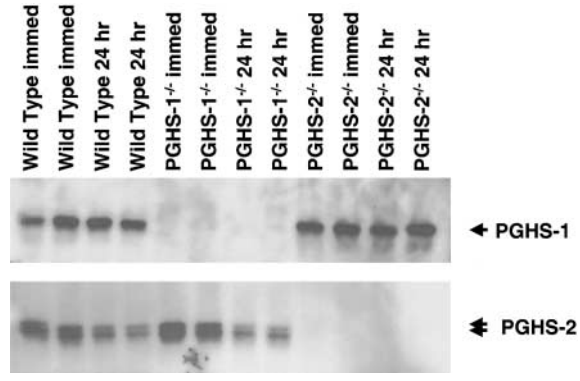

Figure 7. PGHS-1 and PGHS-2 expression in PGHS-$1^{-/-}$, PGHS-$2^{-/-}$, and WT mice exposed to LPS. Mice were exposed to 4 to 6 μg/m^3 LPS via aerosol for 4 h. Immediately after LPS exposure or 24 h later, lungs were frozen, homogenized, and used for Western blotting with PGHS-1– and PGHS-2–specific antibodies. Data shown are representative of results from 9 to 12 animals per group.

tween PGHS-deficient mice and WT controls in total respiratory system resistance, compliance, or responsiveness to MCh (15). In agreement with the results of the current study, we previously observed that after allergen challenge, sensitized PGHS-1$^{-/-}$ mice exhibit increased total respiratory system resistance and MCh hyperresponsiveness compared with WT mice (15). Thus, PGHS-1$^{-/-}$ mice appear to have an exaggerated bronchoconstrictive response to both inhaled allergen and LPS. In contrast, allergen-exposed PGHS-2$^{-/-}$ mice were no different from WT controls in terms of baseline total respiratory system resistance and MCh responsiveness (15), suggesting that the lung functional response of these mice may differ depending on the stimulus.

Perhaps the most intriguing aspect of the current study is that the enhanced bronchoconstrictive response to inhaled LPS in both PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice occurred in the absence of an augmented airway inflammatory response. Thus, there were no significant differences in BALF cells or lung histopathology between the three genotypes immediately after LPS inhalation. Moreover, the levels of several proinflammatory cytokines (TNF-α, IL-6) and chemokines (MIP-2) tended to be lower in LPS-exposed PGHS-deficient mice than in WT controls. Importantly, the extent of airway inflammation and MCh hyperresponsiveness were dissociated in the PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice exposed to LPS. We have previously shown that airway inflammation can be dissociated from the development of AHR in allergen-exposed PGHS-2$^{-/-}$ mice (15). Although airway inflammation is frequently correlated with AHR, and treatment of inflammation often improves this condition, this correlation has not been consistently observed in some studies of animals and humans (25–28).

The lack of an enhanced lung inflammatory response to inhaled LPS in the PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice is interesting given that these mice have an exaggerated lung inflammatory response to inhaled allergen (15). Thus, compared with allergen-exposed WT mice, allergen-exposed PGHS-deficient mice have increased BALF cells, increased histologic evidence for lung inflammation, and increased inflammatory cell activation (15). More recently, we evaluated the response of PGHS-deficient mice to intratracheal instillation of vanadium pentoxide, a metal known to induce severe lung injury in rodents. Remarkably, only the PGHS-2$^{-/-}$ mice had increased lung inflammation after vanadium instillation compared with WT mice; the PGHS-1$^{-/-}$ mice were no different from WT controls (29). Although the features and mechanisms of allergen-, LPS-, and vanadium-induced lung inflammation are different, these data do suggest that the inflammatory responses of PGHS-deficient mice vary depending on the stimulus. Interestingly, Langenbach and coworkers have shown that PGHS-1$^{-/-}$ animals exhibit decreased AA-induced ear inflammation, whereas PGHS-2$^{-/-}$ mice show normal responses to this stimulus (13). Thus, it appears as though the relationship between PGHS expression and inflammation also depends on the model used. Together, the results of these studies suggest caution in the interpretation of our data.

We observed that BALF PGE_2 concentrations were significantly reduced in both PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice compared with WT mice immediately after LPS exposure. These data indicate that both PGHS isoforms contribute significantly to airway PGE_2 production after inhalation of LPS. Because PGE_2 is a potent bronchodilator (9, 30, 31), these results may also explain, at least in part, the exaggerated bronchoconstrictive response to inhaled LPS in the PGHS-deficient mice. Multiple factors may influence BALF PGE_2 levels after LPS exposure, including changes in PGHS-1 or PGHS-2 protein expression, changes in PGHS-1 or PGHS-2 activity, and/or alteration in availability of the substrate, AA. We observed increased lung PGHS-2 protein expression in LPS-exposed PGHS-1$^{-/-}$ and WT mice. Interestingly, LPS induced PGHS-2 protein to a greater extent in PGHS-1$^{-/-}$ mice than in WT mice. This suggests the possibility that PGE_2 and/or other PGHS-1–derived eicosanoids may limit LPS-induced upregulation of PGHS-2. These results are distinctly different from those observed after allergen exposure. In that model, PGHS-2 was induced to a greater extent in WT mice compared with PGHS-1$^{-/-}$ mice, suggesting that PGHS-1 products may be necessary for optimal induction of PGHS-2 to occur (15). We have also observed that vanadium pentoxide increases PGHS-2 expression to a greater extent in WT versus PGHS-1$^{-/-}$ mice (29). Thus, the magnitude and possibly mechanism(s) of PGHS-2 upregulation in PGHS-1$^{-/-}$ and WT mice likely vary depending on the stimulus.

The LTs, products of the 5-LO pathway, are generally considered to be proinflammatory lipid mediators that cause bronchoconstriction and AHR (7, 22, 23, 25). Recent studies with 5-LO$^{-/-}$ mice have shown marked reductions in allergen-induced airway inflammation and bronchial responsiveness (32, 33). Theoretically, disruption of the *Pghs-1* or *Pghs-2* genes might lead to increased cellular AA availability and subsequent "shunting" down the 5-LO pathway, resulting in increased LT biosynthesis. Indeed, we observed increased levels of LTB_4 and sulfidopeptide LTs in BALF from allergen-exposed PGHS-1$^{-/-}$ mice compared with WT controls (15). Similarly, Goulet and associates have reported enhanced release of PGE_2 in peritoneal macrophages from 5-LO$^{-/-}$ mice *in vitro* (34). In contrast, we found here that BALF LTB_4 levels were not significantly different between LPS-exposed PGHS-deficient and WT mice. Thus, it is unlikely that the observed increase in bronchoconstriction and MCh responsiveness in the PGHS-deficient mice involves increased LT production. These data also suggest that "shunting" of AA down the 5-LO pathway in PGHS-deficient mice, if it actually occurs, may occur in a stimulus-specific fashion.

It is now well established that the production of a wide array of cytokines in response to proinflammatory stimuli is regulated by lipid mediators. For example, PGE_2 has been shown to inhibit LPS-induced TNF-α production in a variety of different cell types (35–37). Moreover, we have recently found that PGHS-deficient mice have increased levels of IL-5 and IL-13 in BALF after allergen challenge (Carey, Germolec, and Zeldin, unpublished observation). Surprisingly, we found that LPS-exposed PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ mice had *lower* BALF levels of TNF-α, IL-6, and MIP-2 compared with WT mice. These differences, which do not appear to be due to alterations in lung inflammatory cell influx or alveolar epithelial permeability

between the genotypes, suggest an overall stimulatory effect of PGHS-derived eicosanoids on production of these mediators. Consistent with our data, PGE_2 has been reported to augment IL-6 and IL-10 production by activated macrophages (35, 38).

PenH is an indirect measure of airflow obstruction and can be affected by several variables, including airway tone, lung and chest wall compliance, changes in breathing pattern, and nasal resistance (19, 39). Measurement of airway responsiveness to inhaled MCh by barometric whole-body plethysmography has previously been shown to be a valid indicator of AHR after allergen challenge in mice (39). This method was chosen to estimate airway responsiveness after LPS exposure in the current study because of our interest in evaluating the complex relationships between airway function and airway inflammation. Moreover, we wanted to evaluate differences in lung function between PGHS-deficient and WT mice over time, something that is not possible using invasive methods in tracheostomized, ventilated mice. Given the limited availability of PGHS-deficient mice, it was not feasible to conduct invasive lung function studies after LPS exposure to confirm that, as in the case of allergen exposure, PenH correlates well with measurements of pulmonary resistance. As a result, we suggest caution in interpretation of our airway function data.

In summary, we have demonstrated that compared with WT mice, both PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice exhibit increased bronchoconstriction and enhanced bronchial responsiveness to inhaled MCh immediately after LPS inhalation. There are no significant differences in BALF inflammatory cells or lung histopathology between the three genotypes after LPS exposure, despite reduced BALF cytokines/chemokines and PGE_2 in PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice. LPS induces PGHS-2 protein more in PGHS-$1^{-/-}$ mice compared with WT mice. On the basis of these data, we conclude that: (*1*) airway inflammation and BHR are dissociated in PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice exposed to LPS; and (*2*) the balance of PGHS-1 and PGHS-2 is important in regulating the functional respiratory responses to inhaled LPS; and (*3*) neither PGHS-1 nor PGHS-2 is important in regulating basal lung function or the inflammatory responses of the lung to inhaled LPS. We postulate that studies with PGHS-$1^{-/-}$ and PGHS-$2^{-/-}$ mice will provide insight into the pathogenesis and mechanisms operative during the development of LPS-induced airway disease in humans.

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